The conserved portion of the putative virulence region contributes to virulence of avian pathogenic Escherichia coli

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Colibacillosis is a common systemic disease of worldwide economic importance in poultry, caused by Escherichia coli. E. coli are normally found in the intestines of poultry, but some strains are able to cause extraintestinal disease. Plasmid pVM01 is essential for virulence in avian pathogenic Escherichia coli (APEC) strain E3 in chickens after aerosol exposure and contains the virulence-associated genes iucA, iss and tsh in distinct regions. The determination of the complete sequence of this plasmid identified many ORFs that were highly similar to genes found in the APEC O1 plasmid, as well as many hypothetical ORFs. Truncated versions of pVM01 were constructed and introduced into avirulent APEC strain E3/2.4 and the pathogenicity of these strains was assessed by aerosol exposure. The function of the region of pVM01 that contains the genes for conjugation was confirmed. Strains carrying the truncated plasmids appeared to be of intermediate virulence compared to the wild-type APEC strain E3. The conserved portion of the putative virulence region was found to contribute to the colonization of and generation of lesions in the air sacs. Both the conserved and variable portions of the putative virulence region were shown to contribute to the colonization of the trachea, but the variable portion of the putative virulence region was not required for the strain to confer a virulent phenotype. These results reveal that deletion of the conserved portion of the putative virulence region, but not the variable portion of the putative virulence region, is associated with a decrease in virulence of APEC.

INTRODUCTION

Escherichia coli are found in the normal intestinal microflora of humans and animals, including birds, but specific pathogenic types have been implicated in a number of extraintestinal diseases, including urinary tract infections of humans and respiratory disease of poultry. Avian pathogenic E. coli (APEC) is an economically important pathogen of chickens worldwide and is responsible for increased mortalities in poultry flocks (Barnes et al., 2008). The most common type of colibacillosis is a complex respiratory disease syndrome that primarily affects young birds (Barnes et al., 2008). The pathogenesis of APEC has not yet been fully elucidated, although recent progress in identifying potential virulence factors encoded by these bacteria (Dozois et al., 2003; Germon et al., 2005; Kariyawasam et al., 2006; La Ragione & Woodward, 2002; Li et al., 2005; Schouler et al., 2004) has led to a better understanding of the disease.

Possession of mobile genetic elements such as pathogenicity islands or virulence plasmids is known to contribute to the virulence of bacterial pathogens (Chouikha et al., 2006; Hacker et al., 1997; Ideses et al., 2005; Parreira & Gyles, 2003; Schmidt & Hensel, 2004; Skyberg et al., 2006). Virulence is likely to be multigenic, with the genes present within these genetic elements influencing the pathogenicity of these bacterial strains. Previous studies have found that many APEC isolates carry large plasmids (Doetkott et al., 1996), some of which encode potential virulence factors (Ike et al., 1992), including adhesins such as the temperature-sensitive haemagglutinin, iron-scavenging mechanisms such as aerobactin production, serum resistance, and colicin V (ColV) production. A 93 kb putative virulence region has been identified in pAPEC-O2-ColV, which Johnson et al. (2006b) proposed could be split into ‘conserved’ and ‘variable’ portions based on gene prevalence data from 595 APEC isolates. The conserved region contains the sitABCD and aerobactin operons, and the iroA
gene cluster (Johnson et al., 2006b), all of which have been shown to contribute to the virulence of APEC strain \( \gamma 7122 \) (Dozois et al., 2003; Sabri et al., 2008) after inoculation into air sacs.

The pathogenicity of multiple APEC strains has been assessed using models such as the embryo lethality assay (ELA) or by intravenous (i.v.), subcutaneous (s.c.), intratracheal (i.t.) or aerosol administration of the bacteria to hatched birds. Gibbs et al. (2004) found that the ELA and i.v. and s.c. inoculations were similar in their ability to discriminate between virulent and avirulent APEC; however, the results from i.t. inoculation did not correlate significantly with these models. The aerosol-exposure method developed by Ginns et al. (1998) better approximates the natural route of infection, and allows colonization of the upper respiratory tract to be assessed, but results from it cannot be directly compared to other models, as the same strains were not used.

Strain E3 is an O non-typable: H28 field isolate, isolated in pure culture from the pericardium of a 40-day-old broiler chicken with colibacillosis (Ginns et al., 1998). It is a virulent strain of avian \( E. coli \) that contains six plasmids and produces a colicin(s) and a hydroxamate siderophore. Plasmid curing by subculturing at high temperatures led to the derivation of a series of strains with different plasmid combinations. Using aerosol exposure (Ginns et al., 1998) to examine the pathogenicity of plasmid-cured E3 strains, it was found that the loss of the largest plasmid, pVM01, was correlated with the loss of virulence and an inability to colonize the respiratory tract (Ginns et al., 2000). The introduction of a TnphoA-tagged pVM01 plasmid into the plasmid-cured, avirulent strain E3/2.4 restored the virulent phenotype (Ginns et al., 2000).

The size of pVM01 has been calculated to be approximately 160 kb and it is known to contain the virulence-associated genes \( iucA, tsh \) and \( iss \) (Tivendale et al., 2004). A restriction map of the plasmid has been determined (Tivendale et al., 2004) and these virulence-associated genes mapped to three distinct regions.

The aim of this study was to identify the groups of genes present on pVM01 that are likely to contribute to virulence and to define the regions of this plasmid that are necessary to confer a virulent phenotype.

**METHODS**

**Bacterial strains.** The \( E. coli \) strains used in this study were field isolate E3 and the plasmid-cured strain E3/2.4 (Ginns et al., 2000). The other strains were derivatives of these and their construction is detailed below. The pathogenicity of E3 and the plasmid-cured strain E3/2.4 was determined previously using an aerosol-exposure method (Ginns et al., 2000). The plasmid profiles and virulence-associated genes of the strains are shown in Table 1. All strains were grown in Luria–Bertani (LB) broth or on LB agar at 37 °C overnight with the appropriate antibiotic selection (50 \( \mu \)g ampicillin ml\(^{-1}\) and/or 50 \( \mu \)g kanamycin ml\(^{-1}\)) unless otherwise stated.

**Preparation of plasmid DNA.** DNA from plasmid pVM01::TnphoA and derivatives thereof were prepared using the Qiagen Large Construct kit as recommended by the manufacturer, except that the volume of \( E. coli \) culture was doubled to 1 l and doubled volumes of the lysis buffers were used. The purified plasmids were visualized by PFGE.

**PFGE.** PFGE was carried out in a CHEF-DR III System (Bio-Rad). Plasmids were separated in 1.0 % (w/v) DNA–grade agarose (Progen) in 0.5 x TBE buffer (1 x TBE is 90 mM Tris, 90 mM boric acid and 2 mM EDTA). A lambda ladder (Bio-Rad) and \( HindIII \)-digested lambda DNA were used as molecular mass standards. Electrophoresis was carried out for 20 h at 6 V cm\(^{-1}\) with a switch time of 1–20 s and an included angle of 120° to separate plasmids, or for 11 h with a switch time of 1–6 s to visualize restriction endonuclease digestion fragments. Gels were stained in 1 x TBE containing 0.5 mg ethidium bromide ml\(^{-1}\) for 30 min then destained in distilled H\(_2\)O for 30 min. DNA was visualized by UV transillumination.

**Sequencing pVM01.** The virulence plasmid pVM01 was sequenced using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and a primer-walking approach. DNA sequencing was performed as half-reactions with 4.0 \( \mu \)l Terminator Ready Reaction Mix in a 20 \( \mu \)l reaction containing 5 pmol of the sequencing primer in 1 x Big Dye Terminator v3.1 Sequencing Buffer (Applied Biosystems). Purified plasmid DNA (approx. 500 ng per reaction) or purified PCR products (50–100 ng per reaction) were used as sequencing template. Sequencing of large plasmid template DNA was performed using a Bio-Rad iyclicr PCR thermocycler by incubating the reactions through a rapid thermal ramp of 1 °C s\(^{-1}\) to 96 °C, then 96 °C for 5 min followed by 50 cycles of a rapid thermal ramp of 1 °C s\(^{-1}\) to 96 °C, then 96 °C for 30 s, a rapid thermal ramp of 1 °C s\(^{-1}\) to 50 °C, then 50 °C for 10 s, then 96 °C for 2 s to visualize restriction endonuclease digestion fragments. The extension products were purified using ethanol/EDTA/sodium acetate precipitation to remove unincorporated dNTPs according to the manufacturer’s instructions (Applied Biosystems). Purified extension products were run on an ABI 3130xl Genetic Analyzer (Applied Biosystems) by the Department of Pathology at The University of Melbourne. The sequences obtained were assembled into contiguous sequences using the Vector NTI program ContigExpress (Invitrogen).

**Construction of truncated plasmids.** TnphoA-tagged pVM01 DNA was digested with the restriction endonucleases \( NcoI, SpeI \) and \( XbaI \) (New England Biolabs) at 37 °C for 3 h. After inactivating the enzymes at 65 °C for 20 min, the DNA fragments were ligated using T4 DNA ligase (Promega). The religated DNA was used to transform \( E. coli \) DH5x cells by electroporation (Gene Pulser; Bio-Rad). Transformants were selected on LB agar containing kanamycin. Kanamycin-resistant transformants were then screened by multiplex PCR to confirm their virulence gene profile.

**APEC multiplex PCR.** A multiplex PCR that amplified the virulence-associated genes \( iucA, iss \) and \( tsh \), as well as the 16S rRNA gene, was established. The primers have been published previously (Tivendale et al., 2004), but whilst optimizing the multiplex PCR the forward primer to amplify the \( iucA \) gene was replaced with \( 5'\) - CCTTCCACCGCTGCACCTAAG-3'. To yield a PCR product of 985 bp, the multiplex PCR was performed using 1.25 U Taq DNA polymerase (Promega) in a 25 \( \mu \)l reaction containing 1 x Mg-free buffer, 2 mM MgCl\(_2\), 200 \( \mu \)M each of dNTP, 0.2 \( \mu \)M of the \( iucA \) primers, 0.1 \( \mu \)M of the \( iucA \) primers and 50 nM of the \( tsh \) and 16S rRNA primers. Colonies were selected directly from an agar plate and placed in the PCR. The PCR was incubated at 94 °C for 3 min, then at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min for 26 cycles, then finally at 72 °C for 10 min. PCR products were separated in 2 % (w/v) agarose gels containing 0.5 x TPE (1 x TPE is 30 mM
number plasmids were also used. Plasmids were separated by PFGE.

Restriction endonuclease digestion of truncated plasmids. Restriction endonuclease digestion of truncated plasmid DNA with NotI, SpeI and XhoI was carried out according to the manufacturer’s instructions. The digested DNA was separated by PFGE and the sizes of the DNA fragments were calculated using Kodak Digital Science 1D Image Analysis Software.

Construction of the hybridization probes. Hybridization probes were constructed using PCR products derived from the three virulence-associated genes (*iucA, tsh, iss*). DNA probes were labelled with [α-32P]dATP (Perkin Elmer) by capillary transfer (Sambrook et al., 1989). DNA as previously described (Tivendale et al., 2004), was used as a molecular mass marker.

Southern blot hybridization. Following PFGE of plasmid fragments, DNA was transferred from the gel to nylon membrane (Hybond-N +, Amersham) by capillary transfer (Sambrook et al., 1989). DNA probes were labelled with [α-32P]dATP (Perkin Elmer) using a random-primed DNA labelling kit (Roche). Hybridization was performed in Church buffer (0.5 M Na2HPO4 pH 7.4, 7 % SDS, 1 mM EDTA, 1 % BSA) overnight at 58 °C. Membranes were washed in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) containing 0.1 % SDS at 58 °C three times for 30 min each and autoradiographed with Kodak BioMax MS film at −70 °C.

Bacterial conjugation. Suspensions of both the donor and recipient strains were made by scraping colonies from LB agar plates and resuspending the cells in PBS. Mating was carried out by mixing the suspensions on LB agar at a ratio of 1:1 and incubating at 37 °C for 5–6 h. The mating mix was scraped from the agar and resuspended in PBS. Conjugants were selected on LB agar containing ampicillin and kanamycin. Ampicillin- and kanamycin-resistant conjugants were examined by multiplex PCR and plasmid profile analysis.

Plasmid profile analysis. Plasmid DNA from transformants of E3/2.4 carrying the truncated plasmids was prepared using the Qiagen Plasmid Midi kit as recommended by the manufacturer. The modifications recommended to obtain higher yields of low-copy-number plasmids were also used. Plasmids were separated by PFGE.

Preparation of cultures for pathogenicity testing. A 100 ml volume of Nutrient Broth no. 2 (Oxoid) was inoculated with a single colony and incubated at 37 °C for 24 h. The optical density of the culture at 600 nm was determined. An OD600 of 1.9 corresponded to approximately 5.8 × 10^8 c.f.u. ml−1. The E. coli cultures were centrifuged at 3600 g for 10 min and the cell pellets were resuspended in fresh nutrient broth to yield a concentration of 1–5 × 10^9 c.f.u. ml−1.

Pathogenicity testing. The virulence of the E. coli strains was assessed using the aerosol-exposure method previously used to determine the virulence of APEC strain E3 (Ginns et al., 1998). Briefly, 1-day-old specific-pathogen-free White Leghorn hybrid chicks were given infectious bronchitis virus vaccine (Vic S, Fort Dodge) at ten times the immunizing dose by eyedrop and were then exposed to an aerosol of E. coli for 20 min. Birds were further exposed to aerosols of E. coli on days 4 and 7, and surviving birds were killed on day 11 by exposure to halothane. The air sacs and tracheas of each bird were swabbed and the swabs were used to inoculate MacConkey agar. Plates were incubated at 37 °C overnight. The number of colonies was counted (1–300) or estimated (>300) and the identity of typical colonies confirmed by multiplex PCR and antimicrobial sensitivity testing. Negative control birds were given an aerosol of nutrient broth instead of E. coli. The percentage weight gain of the birds was calculated, and the mean determined for each experimental group.

At post-mortem, birds were examined for air-sacculitis, pericarditis, peripheratitis and peritonitis. Air sacs were scored from 0 (no lesions) to 4 (severe opacity in the membranes and caseous exudate) for both the left and right, anterior and posterior thoracic and abdominal air sacs.

Antimicrobial sensitivity testing. The antimicrobial sensitivity of the E. coli strains was assessed using the calibrated dichotomous sensitivity test of Bell (1984) on Sensitest agar (Oxoid). An annular radius of >6 mm indicated that the organism was sensitive to the antimicrobial agent while a radius <6 mm indicated resistance. Antimicrobial discs (Oxoid) used were ampicillin 25 μg, sulfafurazole 300 μg, trimethoprim 5 μg, chloramphenicol 30 μg, tetracycline 30 μg and kanamycin 50 μg.

Statistical analysis. Fisher’s exact test was used to compare lesion and resolation rates, Student’s t-test to compare mean body weights, and the Mann–Whitney test to compare differences between the lesion scores and bacterial numbers isolated from each group.

pVM01 sequence. The complete sequence of pVM01 was deposited in GenBank under the accession number EU330199.

### RESULTS

#### Sequencing of pVM01

Using the virulence-associated genes known to be present on pVM01, namely *iucA, iss, tsh, sitA* and *iroB*, plasmid

### Table 1. Plasmid profiles, phenotypic characteristics and virulence genes of the E3 derivatives

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmids</th>
<th>Virulence-associated genes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3</td>
<td>pVM01, pVM02, pVM03, pVM04</td>
<td><em>iucA, tsh, iss</em></td>
</tr>
<tr>
<td>E3/2.4</td>
<td>pVM02, pVM03</td>
<td>None detected</td>
</tr>
<tr>
<td>E3/2.4/I</td>
<td>pVM01::TnphoA, pVM02, pVM03</td>
<td><em>iucA, tsh, iss</em></td>
</tr>
<tr>
<td>E3/2.4/NorAC</td>
<td>pVM01::TnphoA (NorAC), pVM02, pVM03</td>
<td><em>iucA, iss</em></td>
</tr>
<tr>
<td>E3/2.4/SpeA</td>
<td>pVM01::TnphoA (SpeA), pVM02, pVM03</td>
<td>tsh</td>
</tr>
<tr>
<td>E3/2.4/SpeAB</td>
<td>pVM01::TnphoA (SpeAB), pVM02, pVM03</td>
<td><em>iucA, tsh</em></td>
</tr>
<tr>
<td>E3/2.4/XhoIA</td>
<td>pVM01::TnphoA (XhoIA), pVM02, pVM03</td>
<td><em>iucA</em></td>
</tr>
<tr>
<td>DH5α</td>
<td>None isolated</td>
<td>None detected</td>
</tr>
</tbody>
</table>

*Identified by PCR amplification.
pVM01::TnphoA DNA was sequenced on both strands using primer walking and Big Dye Terminator chemistry. Approximately 600 sequencing reads, averaging 700 bp in length, were obtained.

Assembly and annotation

Sequence data were assembled using ContigExpress in the Vector NTI package from Invitrogen and full coverage of both the positive and negative strands was achieved, resulting in the generation of one complete contiguous sequence of 151 002 bp.

The TnphoA insert was deleted from the pVM01::TnphoA sequence to produce sequence for pVM01 alone. This was achieved by aligning the pVM01::TnphoA sequence with the 7733 bp TnphoA sequence from the GenBank database (accession number U25548), then deleting the corresponding TnphoA sequence from pVM01::TnphoA. The sequence around the site of the TnphoA insertion was confirmed by PCR amplification of this region of pVM01 from the wild-type strain E3, and subsequent sequencing of the resultant PCR product.

The size of restriction endonuclease fragments of pVM01::TnphoA had been estimated previously (Tivendale et al., 2004), and PFGE determined there were three NotI fragments, three XbaI fragments and four SpeI fragments. Restriction endonuclease cleavage site analysis of the 151 kb pVM01 sequence identified one NotI restriction site, three XbaI restriction sites and seven SpeI restriction sites (Table 2). Only one NotI restriction site was identified in the pVM01 sequence as the two other NotI restriction sites observed after PFGE of restriction endonuclease fragments of pVM01::TnphoA were in the TnphoA insert that was excluded during sequencing of pVM01. As shown in Table 2, there were seven SpeI restriction sites, but three of these were within 316 bp of one another and the resulting restriction fragments would not be observable by PFGE. Similarly, another two SpeI restriction sites were separated by only 12 bp, which would have been impossible to differentiate by PFGE.

ORFs in the plasmid sequence were predicted using GeneMark-P and GeneMark.hmm for Prokaryotes (version 2.4) with E. coli K-12 as the model organism (Lukashin & Borodovsky, 1998). GeneMark-P determines the protein-coding potential of a DNA sequence by using species-specific parameters in hidden Markov models of coding and non-coding regions. Translated ORFs were then compared to known protein sequences using pBLAST (NCBI, August 2007). Proteins with the highest score were recorded as the closest match and when proteins from APEC O1 were amongst those with the highest score, they were preferentially recorded (see supplementary Table S1, available with the online version of this paper). Those with 100 % identity covering 100 % of the matching protein sequence were classified as identical. Translated ORFs with more than 90 % identity covering more than 80 % of the matching protein sequence were classified as highly similar, and those with 50–90 % identity covering more than 80 % of the matching protein sequence were classified as similar. Translated ORFs with less than 50 % identity covering more than 80 % of the matching protein sequence were classified as weakly similar and those covering less than 80 % of the sequence were classified as a partial match. Translated ORFs for which no significantly similar proteins were found in the GenBank database were classified as hypothetical proteins.

The G+C content of the entire pVM01 plasmid was 49.5 mol%. The G+C content of individual ORFs was analysed using GC Calculator (http://www.genomicsplace.com/gc_calc.html) and is shown in the supplementary material. Insertion sequences (ISs) and repetitive elements were identified using IS FINDER (http://www-is.biotoul.fr/is.html) and are shown in Fig. 1.

The 151 kb pVM01 contained 153 predicted ORFs (Table S1). Of these predicted coding regions, 14.4 % (22/153) shared sequence similarity with IS elements. Of the remaining coding regions, 32.7 % (50/153) were found to be an identical match to available database proteins, 43.1 % (66/153) were found to be highly similar to available database proteins, 2.0 % (3/153) were found to be similar to available database proteins, and 2.0 % (3/153) were found to be weakly similar to available database proteins. Only one ORF (0.7 %) shared no significant identity with any available database protein and 5.2 % (8/153) were found to be only a partial match to available database proteins.

A region similar to the virulence region of pAPEC-O2-ColV, of approximately 80 kb, was identified in pVM01 and contained the virulence-associated genes tsh, encoding a temperature-sensitive haemagglutinin (Provence & Curtiss, 1994), iss, encoding increased serum survival (Horne et al., 2000), cvaA and a truncated cvaB from the ColV operon (Gilson et al., 1987), etsABC, a putative ABC

Table 2. Location of restriction endonuclease cleavage sites and the TnphoA insert in pVM01

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<tbody>
<tr>
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<td>57 478</td>
</tr>
<tr>
<td>XbaI cleavage</td>
<td>35 699, 37 826, 90 179</td>
</tr>
<tr>
<td>SpeI cleavage</td>
<td>33 697, 39 960, 39 972, 40 276, 59 156, 59 168, 144 193</td>
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<td>118 558</td>
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transport system recently identified in *E. coli* (Johnson *et al.*, 2006b), and other genes found within the virulence region of pAPEC-O2-ColV, including *shiF*, *hlyF*, *ompT* and the *repA* and *int* genes of the RepFIB replicon. The genes *yadA*, *yacC*, *yacB*, *yacA*, *cbi*, *cma*, *cmi*, *vagC* and *vagD*, present in the putative virulence region identified in pAPEC-O1-ColBM (Johnson *et al.*, 2006a), were also found within this region. Several genes encoding iron-acquisition systems were found within the putative virulence region, including the *iroA* gene cluster, encoding the salmochelin siderophore (Hantke *et al.*, 2003), the *sitABCD* operon, involved in iron and manganese transport (Sabri *et al.*, 2006; Zhou *et al.*, 1999), and the aerobactin operon, encoding the production of the aerobactin siderophore (de Lorenzo *et al.*, 1986). The annotated sequence of pVM01 is presented in Table S1.
beginning with the putative virulence region, and is illustrated in Fig. 1.

A region encompassing the full transfer region was identified adjacent to the putative virulence region. The F-like transfer region of pVM01 spanned approximately 32.8 kb and contained 36 genes. Following the tra and trb genes of the transfer region were the psiA and psiB genes, which are involved in plasmid maintenance and stability, and the remaining sequence consisted of genes mostly encoding hypothetical proteins of unknown function, with the exception of the sopA and sopB genes, which are involved in F plasmid partitioning (Mori et al., 1989).

**IS elements in pVM01**

A total of 14 IS elements were identified in pVM01, including five copies of IS1, two copies of IS2, two copies of IS186, two copies of IS629, a partial copy of IS21, and single copies of IS26 and IS100 (Fig. 1). Inverted IS1 elements flank the sitABCD and aerobactin operons and another set of inverted IS1 elements flank the region containing the tsh gene, facilitating the mobility of these virulence-associated genes and suggesting a mechanism for evolution of these virulence plasmids.

**Alignment of APEC virulence plasmids**

A global alignment of pVM01 and pAPEC-O1-ColBM, using the Stretcher program from Biomanager (http://www.angis.org.au), found that pVM01 had 63.7 % nucleotide identity to pAPEC-O1-ColBM. Only 46.1 % nucleotide identity was observed between pVM01 and pAPEC-O2-ColIV using the same global alignment program. Local alignments of the nucleotide sequence of each pVM01 ORF to both the pAPEC-O2-ColIV and pAPEC-O1-ColBM sequences were generated using the BLAST 2 sequences tool (bl2seq, NCBI) (Tatusova & Madden, 1999). Nucleotide sequence similarity to the pVM01 sequences tool (bl2seq, NCBI) (Tatusova & Madden, 1999).

**Physical characterization of truncated plasmids**

Plasmid pVM01::TnphoA DNA was digested, religated and used to transform E. coli strain DH5α. Transformants were selected for kanamycin resistance and four distinct truncated plasmids were identified by their different virulence gene profiles in multiplex PCR. Restriction endonuclease digestion of these truncated plasmids and Southern blot hybridization identified the fragments that had religated to form the truncated plasmids. The resultant plasmids are illustrated in Fig. 1 and the nucleotide sequences excised from pVM01 during the construction of the truncated plasmids are indicated in Table 3.

The truncated plasmids were transferred by conjugation from the donor strain, DH5α, to the plasmid-cured recipient strain, E3/2.4. The plasmid profiles of the E3/2.4 conjugants are shown in Fig. 2. All four strains carry the strain E3 plasmids pVM02 and pVM03, as well as their respective truncated pVM01::TnphoA plasmid. The XbaIA plasmid cannot be seen on the ethidium bromide-stained gel, but Southern blot hybridization with the TnphoA probe confirmed that the XbaIA plasmid was superimposed on the pVM02 band.

**Pathogenicity testing**

The virulence of E3 has been assessed previously (Ginns et al., 2000) and challenge with E3 was used as the positive control in this study. The results shown in Table 4 detail the lesion rates following aerosol exposure to the different strains.

Air sacculitis was the major lesion caused by the APEC strains, although some birds developed perihepatitis, peritonitis and/or pericarditis. The pathogenicity of the strains was assessed by comparing the mean percentage weight gain, median air sac lesion scores and reisolation rates of APEC from the air sacs and tracheas and the numbers of APEC recovered from swabs taken from air sacs and tracheas of birds in each of the experimental groups.

The mean weight gain in uninfected control birds was 183.5 %, which was significantly higher than the mean weight gains of each of the groups of infected birds (range from 144.4 % to 159.0 %). The mean weight gains were not significantly different between the birds infected with E3 (159.0 %) or conjugants of E3/2.4 (range from 144.4 % to 158.3 %). The mortality rates were minimal and did not differ significantly between groups.

E3/2.4/SpeIA was the only strain that resulted in a significantly lower median lesion score than the wild-type strain, E3 (P=0.018), and E3/2.4/NotIAC was the only

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**Table 3. Sequence excised from pVM01 to create truncated plasmids**

<table>
<thead>
<tr>
<th>Truncated plasmid</th>
<th>Nucleotides excised from pVM01 sequence</th>
<th>Corresponding genes</th>
<th>TnphoA sequence present</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNotIAC</td>
<td>57 478 to 118 558</td>
<td>yacC to traC</td>
<td>First 7134 bp</td>
</tr>
<tr>
<td>pSpeIA</td>
<td>144 193 to 59 168</td>
<td>IS1 after sopA to yacA</td>
<td>All 7733 bp</td>
</tr>
<tr>
<td>pSpeIAB</td>
<td>33 697 to 59 168</td>
<td>Conserved hypothetical after etsC to yacA</td>
<td>All 7733 bp</td>
</tr>
<tr>
<td>pXbaIA</td>
<td>35 699 to 90 179</td>
<td>Conserved hypothetical before IS2 to ydaA</td>
<td>All 7733 bp</td>
</tr>
</tbody>
</table>
strain carrying a truncated plasmid that resulted in a significantly higher lesion score than E3/2.4/SpeIA (P=0.002).

The median numbers of colonies isolated from air sac and tracheal swabs of birds in each group are shown in Table 4.

Significantly fewer APEC were isolated from both the tracheas (P=0.005) and air sacs (P=0.006) of birds infected with E3/2.4/SpeIA than from the birds infected with E3. Significantly fewer APEC were isolated from the tracheas of birds infected with E3/2.4/SpeIA than from the tracheas of birds infected with E3/2.4/XbaIA (P=0.029).

Significantly more APEC were isolated from the air sacs of birds infected with E3/2.4/NotIAC than from birds infected with strain E3 (P=0.035).

**DISCUSSION**

The complete sequence of an APEC virulence plasmid is reported in this study, despite the presence of the numerous IS elements that prevented the closure of the first sequenced APEC virulence plasmid, and resulted in its description as three separate contiguous sequences (Johnson *et al.*, 2006b). The 93 kb putative virulence region found in pAPEC-O2-ColV (Johnson *et al.*, 2006b) and also in pAPEC-O1-ColBM (116 kb) (Johnson *et al.*, 2006a) was found as an 80 kb region in pVM01 in the present study.

The G+C content of pVM01 was 49.5 mol%, almost identical to the 49.6 mol% G+C content of the 174 242 bp pAPEC-O1-ColBM (Johnson *et al.*, 2006a).

Estimation of the size of restriction endonuclease fragments of pVM01 resulted in a calculated size for pVM01::TnphoA of approximately 160 kb (Tivendale *et al.*, 2004), but sequencing of pVM01 accurately determined that its size was 151 002 bp. This is considerably smaller than both of the previously sequenced APEC virulence plasmids, pAPEC-O1-ColBM (174 242 bp) (Johnson *et al.*, 2006a) and pAPEC-O2-ColV (179 495 bp) (Johnson *et al.*, 2006b).

A global alignment between pVM01 and pAPEC-O1-ColBM found 63.7 % nucleotide similarity between the two APEC virulence plasmids, but a global alignment between
pVM01 and the pAPEC-O2-ColV sequence found only 46.1% nucleotide similarity. The incomplete nature of the sequence of pAPEC-O2-ColV, as found in the GenBank database (AY545598), may have contributed to the lower identity observed between pVM01 and pAPEC-O2-ColV.

The putative virulence region in pVM01 was found to start with the *iutA* gene and proceeded through to the *tsh* gene, whilst in pAPEC-O2-ColV it spanned from *siaA* through to *eitD*, and in pAPEC-O1-ColBM from *tsh* to *etsC*. Whilst the orientation of the virulence-associated genes within the virulence region varied within each plasmid, the virulence-associated gene content was consistent between the plasmids. In particular, each of the three plasmids contained the aerobactin and *sitABCD* operons, the *iroA* gene cluster, *etsABC*, *shiF*, *hlyF*, *ompT*, *tsh*, *iss*, and the RepFIIA replicon and these regions had ≥95% nucleotide sequence identity (Fig. 1). This observation is consistent with previous findings that the genes of the conserved portion of the virulence region, including *sitABCD*, *iucABCDiutA*, *iroBCDEN*, *iss*, *hlyF* and *etsABC*, are highly conserved amongst APEC isolates (Johnson et al., 2006b).

Johnson et al. (2006b) suggested that the virulence region was conserved until the *cvaB* gene, after which it became variable. In pVM01, the *cvaB* gene was fused to the IS186 element, and this was followed by a region containing many genes encoding hypothetical proteins, *tsh*, genes of the RepFIIA replicon and the transfer region. The other copy of IS186 was located on the edge of this region and could be considered to be the breakpoint between genes involved in plasmid maintenance and stability and those of the putative virulence region.

Overall, 83.2% (109/131) of the predicted proteins of pVM01, not including IS elements, are also found on pAPEC-O1-ColBM and 53 ORFs from pVM01 that correspond to proteins of known function are also found on pAPEC-O2-ColV. Most of these are genes found in the putative virulence region of pAPEC-O2-ColV. Although pVM01 is similar to the other sequenced APEC virulence plasmids, there are key differences between them.

As shown in Fig. 1, there is a region of approximately 5 kb between the RepFIIA replicon and the transfer region encoding hypothetical proteins that is unique to pVM01.

The *eitABCD* genes, encoding a putative ABC iron-transport system, found in both pAPEC-O2-ColV (Johnson et al., 2006b) and pAPEC-O1-ColBM (Johnson et al., 2006a), were not found on pVM01. Johnson et al. (2006b) described the portion of the virulence region containing the *eitABCD* genes as variable, which may explain their absence from the virulence region of pVM01.

The ORF identified as *traG* in pVM01 is only a partial match to *traG* in pAPEC-O1-ColBM and the ORF identified as *traH* in pVM01 is absent from pAPEC-O1-ColBM (Johnson et al., 2006a). The annotation of pAPEC-O1-ColBM identified only one large ORF, identified as *traG*, encompassing both *traG* and *traH*, as identified in pVM01. Similarly, coding regions for both *traG* and *traH* were identified in pAPEC-O2-ColV (Johnson et al., 2006b).

The majority of the 16.8% of predicted proteins in pVM01 that were not found in pAPEC-O1-ColBM were hypothetical proteins. Many of these hypothetical proteins were found in the region of pVM01 that contained genes involved in plasmid maintenance and stability and, whilst these hypothetical proteins have not been found in the other APEC virulence plasmids, they exhibited similarity to proteins in UPEC and *E. coli* O157:H7.

As in pAPEC-O2-ColV and pAPEC-O1-ColBM, inverted IS1 elements flanked the *sitABCD* and aerobactin operons in pVM01. However, one difference between pAPEC-O2-ColV (Johnson et al., 2006b) and pVM01 was the orientation of this region with respect to the RepFIB region and the remaining virulence-associated genes. In pAPEC-O2-ColV the genes *iutA* and *repA* lie adjacent to one another, whilst in pVM01, *siaA* and *repA* are adjacent to one another, as also seen in pAPEC-O1-ColBM (Johnson et al., 2006a). Many of the virulence-associated genes located in the putative virulence region are framed by insertion sequences, indicating that pVM01 was probably assembled by integration of distinct horizontally acquired DNA segments via transposition or homologous recombination. Interestingly, the mosaic nature of pVM01 is very similar to that of pAPEC-O2-ColV and pAPEC-O1-ColBM, and many of the virulence-associated gene sequences and accompanying insertion sequences are almost identical, suggesting a common ancestor for these virulence plasmids in APEC.

The fragments excised from pVM01::TpnsphoA to create the truncated plasmids (Table 3) contained different areas of the 80 kb putative virulence region. Only the conserved portion of the putative virulence region was present in pNotIAC, whilst it was absent from pSpeIA, which contained only the variable portion of the putative virulence region. The first half of the conserved portion of the putative virulence region, up to the *etsABC* genes, was present in pSpeIAB, as well as the variable portion of the putative virulence region, and pXbaIA contained only the first half of the conserved portion, up to the *etsABC* genes.

As all four truncated plasmids were conjugative, it can be deduced that the conjugative genes of pVM01 were located on the 24.9 kb SpeI–NotI fragment to which the TpnsphoA probe hybridized, as this was the only fragment common to all four truncated plasmids. Sequencing of pVM01 showed that the *sopAB* genes were found on this fragment, along with genes from the beginning of the transfer region up to the start of *traC* (118 567 bp). The genes of the transfer region from *traC* up to *traX* were not present in the conjugative plasmid pNotIAC, indicating that they were not essential for conjugation.

Genes of both the RepFIB and RepFIIA replicons were present in pVM01 and pSpeIAB, but only genes of the
RepFIB replicon were present in pNotIAC and pXbaIA, whilst only genes of the RepFIIA replicon were present in pSpeIA. This indicates that both replicons are capable of replication in pVM01. Genes of both the RepFIB and RepFIIA replicons are also present in both pAPEC-O2-ColIV (Johnson et al., 2006b) and pAPEC-O1-ColBM (Johnson et al., 2006a), and Johnson and co-workers suggested that plasmids possessing more than one replicon may use alternative replication methods to avoid plasmid incompatibility.

In the pathogenicity studies, weight gain appeared to be useful in differentiating infected birds from uninfected birds, but was not useful in differentiating between the varying levels of virulence of the strains. Mortality was not a useful measurement of virulence.

Conjugants of strain E3/2.4 carrying truncated versions of the virulence plasmid pVM01 displayed varying levels of virulence, but none were avirulent. Taking into account all measures of virulence, E3/2.4/SpeIA appeared to be the least pathogenic of the conjugants carrying the truncated plasmids. The SpeIA plasmid lacked the majority of the putative virulence region of pVM01, and contained only the segment from cvaA through to tsh, which has previously been described as the variable portion of the virulence region. The lower level of virulence observed for the strain carrying this plasmid, which lacked the conserved portion of the putative virulence region, was consistent with the predictions of Johnson et al. (2006b).

Carriage of plasmid pNotIAC, which contained the conserved portion of the putative virulence region, including the aerobactin and sitABCD operons and the iroA cluster, appeared to result in increased colonization of the air sacs, as well as an increased ability to cause lesions in the air sacs, compared to carriage of plasmid pXbaIA, which lacked the genes from nt 35 699 to 90 179, including the last 25 kb of the conserved portion and the variable portion of the putative virulence region. This suggests the region of pVM01 from nt 35 699 to 57 478, which corresponds to the second half of the conserved portion of the putative virulence region and contains iss and the iroA cluster, in addition to the first half of the conserved portion of the putative virulence region, is involved in colonization of and generation of lesions in the air sacs.

The only genes in plasmid pSpeLAB that were not in pSpeIA were contained in a 39.5 kb fragment and included the aerobactin operon, shiF, the sitABCD operon, repA, int, hlyF, ompT and etsABC. Thus, as significantly more APEC were isolated from the air sacs of birds infected with E3/2.4/SpeLAB than from those of birds infected with E3/2.4/SpeIA, it appears that the 39.5 kb SpeIB fragment increases the capacity for colonization of the air sacs, which correlates with a study showing a reduction in invasion of various tissues after inoculation directly into the air sacs in mutants of APEC strain γ7122 from which the sitABCD operon has been deleted (Sabri et al., 2008). Many of the genes contained within the 39.5 kb fragment are found within the first 34 kb of the conserved portion of the putative virulence region of pVM01 and are also found in the putative virulence region of pAPEC-O2-ColIV (Johnson et al., 2006b) and pAPEC-O1-ColBM (Johnson et al., 2006a), reinforcing the inferred role of this region in the virulence of APEC.

The wild-type APEC, E3, colonized the trachea of the birds to a greater extent than the air sacs. However, in birds infected with E3/2.4/NotIAC, which lacked the sequence from nt 57 478 to 118 558, containing the variable portion of the putative virulence region including the tsh gene, there was greater colonization of the air sacs than the trachea. This corresponded with a greater severity of air-sac lesions in birds infected with this strain. This suggests that tsh, or another gene found on the NotIB fragment, enhances colonization of the trachea, an observation consistent with a previous study that suggested that tsh was involved in the initial stages of colonization (Provence & Curtiss, 1994). It may be that if APEC are unable to adhere to the trachea, they concentrate in the air sacs, resulting in more severe air sacculitis.

Ginns et al. (2000) found that pVM01 was necessary for colonization of the upper respiratory tract and the study reported here has further defined the regions of pVM01 involved in colonization of the trachea, the loss of the fragment containing tsh correlating with the reduced ability of the strain to colonize the trachea, but not the air sacs.

Comparison of the pathogenicity of the strain carrying plasmid pXbaIA, containing the conserved portion of the putative virulence region including the aerobactin and sitABCD operons, with that carrying plasmid pSpeIA, containing the variable end of the putative virulence region including the tsh gene, found significant differences only in the numbers of APEC reisolated from the trachea, with the strain carrying the conserved segment of the putative virulence region colonizing the trachea to a greater extent than the strain containing only the variable region. It is therefore possible that the conserved portion of the putative virulence region, in addition to the variable region, is required for optimal colonization of the trachea.

Strains E3/2.4/XbaIA, containing the first 36 kb of the conserved region, and E3/2.4/SpeLAB, containing the variable region in addition to the first 34 kb of the conserved region, did not differ significantly in virulence. This suggests that the variable portion of the putative virulence region, including the tsh gene, does not greatly contribute to the virulence of APEC. This reinforces previous findings suggesting that tsh does not play a major role in the pathogenicity of APEC strain E3 (Tivendale et al., 2004) and possibly APECs in general.

The findings of this study are consistent with those examining the virulence of APEC strain γ7122 after deletion of the aerobactin and sitABCD operons or the iroA cluster (D佐ois et al., 2003; Sabri et al., 2008), but contrast with the findings of Skyberg et al. (2008), who did
not detect any difference in virulence between mutants lacking genes in the putative virulence region and the wild-type strain. It is probable that these differences are attributable to the considerable differences in the infection models used to assess virulence. It is notable that none of these studies would have been able to detect the differences we have found in the capacities of mutants to colonize the upper or lower respiratory tracts, and the correlation between these capacities and the induction of air sacculitis, because in all cases the bacteria were inoculated directly into the birds, bypassing the upper respiratory tract, and in the study of Skyberg et al. (2008), bypassing the respiratory tract completely. Thus significant events in the pathogenesis of colibacillosis, and the roles the specific genes may play in these events, may be overlooked unless a model incorporating aerosol exposure is used.

In conclusion, this study has assessed the contribution of large fragments of pVM01 to virulence and the findings will facilitate studies utilizing specific gene knockouts, targeted at the regions found to be most significant in studying different aspects of the pathogenesis of colibacillosis, to further enhance our understanding of the contribution of the different virulence-associated genes to the pathogenicity of APEC.

ACKNOWLEDGEMENTS

This work was funded by the Australian Poultry CRC.

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Edited by: B. Kenny